Supplement

Supplementary Methods

Patients whose samples were used in this study were treated at John's Hopkins University, the University of California San Francisco, or the University of Pennsylvania on a phase II or III trial of quizartinib monotherapy[10-11] for patients with relapsed or refractory AML. Analysis was conducted on samples from the time of study entry and at the time of relapse on quizartinib monotherapy. Samples were collected in accordance with the Declaration of Helsinki under institutional review board-approved tissue banking protocols, and written informed consent was obtained from all patients. Patients were selected if they relapsed after initial treatment with quizartinib monotherapy and had serial samples available for analysis. Samples were distinct from those used in our previous quizartinib analysis[18].

We performed single cell (SC) DNA sequencing on unsorted mononuclear cells using the Tapestri platform (Mission Bio Inc). This platform's technology utilizes a "two-step" droplet-based microfluidics workflow[12]. Cells are first encapsulated and lysed, and then chromatin/protein complexes are digested with proteases. After heat inactivation of the proteases, molecular barcodes and PCR reagents are added via microfluidics to the lysate drops containing single-cell nucleic acids. Droplets are thermocycled and the barcodes are incorporated into amplicons from multiple genomic loci. For this set of patients, targeted sequencing of mutational hotspots included 40 amplicons from 19-AML specific genes plus 10 amplicons to control for allele drop out. The DNA was then incorporated into a library preparation workflow similar to that used for other next generation sequencing applications, including purification and PCR amplification via AmpureXP (Beckman Coulter). DNA was quantified using the Qubit Fluorometer (ThermoFisher) and library size was measured with the high-sensitivity Bioanalyzer 2100 DNA Assay (Agilent Technologies). Libraries were normalized, pooled, and sequenced using 150 pair end reads on a HiSeq4000 (Illumina).

To analyze the data, paired-end FASTQ files, generated by the Illumina HiSeq4000, were processed by two different analysis pipelines: the commercially available Tapestri pipeline (Mission Bio Inc.) and a non-commercial variant calling pipeline utilizing GATK best practices workflows[14,21]. In both cases, high quality reads were demultiplexed for cell calling using cell-specific barcodes, single cells were filtered based on read depth and distribution, reads were aligned to the reference genome hg19 (BWA), and variants were called using GATK3.7/HaplotypeCaller. Variants selected for downstream analysis were identified by qualitative variant annotation information (e.g., ClinVar) as well as quantitative pathogenicity metrics (e.g., Dann). Candidate pathogenic mutations were manually reviewed via Integrative Genomics Viewer[15] via the non-commercial pipeline. Internal tandem duplications (ITDs) were specifically identified by a custom algorithm (Mission Bio Inc.): if there were more than ten reads with more than four non-reference reads and a ratio of non-reference to reference reads greater than 0.1, the cell was considered to have a non-reference or alternate allele. If the ratio of non-reference to alternative alleles was greater than a preset cutoff (0.9), it was considered to be homozygous. Based on variant call data and determined cell populations, single cell phylogenies and populational hierarchies were reconstructed. Included figures represent one possible evolutionary trajectory based on detectable mutational data.

All SCS data is deposited into dbGAP.

Supplementary Tables and Figures

Supplementary Table 1. Additional Patient Clinical Data.

Patient ID	Age	Sex	Cytogenetics at Study Entry	Cytogenetics at Relapse if Different Than Baseline	Dose of quizartinib
1	32	M	46,XY,del(5)(q23q33)[5]/46,XY[4]	46,XY,del(5)(q23q33)[14]/46,XY[1]	135mg
2	65	M	47,XY,+11[15]	47,XY,+11[13]/47,sl,add(17)(p11.2)[9]	135mg
3	64	F	46, XX		30mg x 2 months, 60mg 2 weeks
4	69	F	46, XX		30mg x 5 weeks, 60mg x 7 weeks
5	70	M	unavailable	46,XY,del(20)(q11.2)[2]/46,XY[21]	30mg x 8 weeks, 60mg x 2 weeks
6	38	F	47, XX, +8, t(x;10)		30mg
7	59	F	47,XX,+8[1]/47,idem,del(16)(q13)[19]	47,XX,+8,del(16)(q13)[20]	90mg
8	45	F	47,XX,+8[3]	47,XX,+8[3]/47,sl,del(11)(q21q23),t(16;19)(q22;p13.3)[14]/46,XX[3]	30mg x 5 weeks, 60mg x 1 week

Supplementary Table 2. Variant Allele Frequencies (VAFs) by aggregate bulk sequencing compared to single cell sequencing (SCS)-derived population frequencies. SCS illuminates more complicated clonal architecture and can directly measure zygosity and co-mutations.

Patient 1

	Time Points	WT	FLT3 ITD #1	FLT3 ITD #2	NRAS G13D	NRAS Q61R	KIT D816V
VAF % by bulk sequencing	Pre-quizartinib		54.10	7.58	0.08	0.04	0.17
	Relapse		0.62	0.26	6.11	50.10	1.41
Population frequency % by SCS	Pre-quizartinib	16.65	72.78	10.20	0.11	0.05	0.23
	Relapse	14.45	0.89	0.38	8.83	73.41	2.04

Patient 2

	Time Points	WT	FLT3 D835Y*	FLT3 D835V	FLT3 N841K	KRAS G13D*	KRAS G13D homo-zygous*	FLT3 D835Y homo-zygous*
VAF % by bulk sequencing	Pre-quizartinib		0.00	0.00	5.60	1.00		
	Relapse after quizartinib		37.90	6.00	0.90	5.20		
	Relapse after quizartinib + chemotherapy		31.10	1.00	1.80	0.60		
Population frequency % by SCS	Pre-quizartinib	89.77	0.00	0.00	10.07		0.17	0.00
	Relapse after quizartinib	7.82	74.22	10.34	1.05		4.83	1.74
	Relapse after quizartinib + chemotherapy	27.58	60.02	0.69	9.92		0.20	1.59

*Bulk sequencing cannot determine zygosity.

Patient 3

	Time Points	WT	FLT3 ITD #1	FLT3 ITD #2	FLT3 D835G*	WT1 R374G*	WT1 R385G*	DNMT3A R882H	WT1 R374G, FLT3 ITD #1*	WT1 R374G, FLT3 ITD #2*	FLT3 D835G, WT1 R374G*
VAF % by bulk sequencing	Pre-quizartinib		9.10	32.00	0.00	23.00	1.35	40.80			
	Relapse		1.00	0.00	41.70	43.40	0.90	46.10			
Population frequency % by SCS	Pre-quizartinib	20.52	6.93	13.56				11.19	3.17	44.63	0.00
	Relapse	9.94	0.00	0.00				2.18	0.00	0.00	87.88

*Bulk sequencing cannot determine co-mutations

Patient 4

	Time Points	WT	FLT3 ITD*	FLT3 D835Y*	FLT3 ITD, FLT3 D835Y**	FLT3 ITD homo-zygous*	FLT3 ITD hetero-zygous*
VAF % by bulk sequencing	Pre-quizartinib		64.00	0.00			
	Relapse		98.00	47.00			
Population frequency % by SCS	Pre-quizartinib	26.28			0.00	20.09	8.94
	Relapse	0.52			45.11	1.22	1.48

*Bulk sequencing cannot determine zygosity
**Bulk sequencing cannot determine co-mutations.

Patient 5

	Time Points	WT	FLT3 ITD*	FLT3 D835Y **	FLT3 D835V **	FLT3 I836S	DNMT 3A R882H	FLT3 ITD hetero-zygous	FLT3 ITD homo-zygous	FLT3 ITD hetero-zygous, D835Y	FLT3 ITD homo-zygous D835Y	FLT3 ITD homo-zygous I836S	FLT3 ITD hetero-zygous D835V
VAF % by bulk sequencing	Pre-quizartinib		62.25	0.00	0.00	0.00	45.90						
	Relapse		74.7	31.20	2.60	7.90	48.90						
Population frequency % by SCS	Pre-quizartinib	3.23					0.38	18.25	10.30	0.00	0.00	0.00	0.00
	Relapse	0.72					0.28	0.98	4.25	11.34	5.88	4.87	0.74

*Bulk sequencing cannot determine zygosity
**Bulk sequencing cannot determine co-mutations.

Patient 6

	Time Points	FLT3 ITD*	FLT3 D835Y**	FLT3 D835H**	WT1 S386 stop **	ASXL1 L815P**	FLT3 ITD homo-zygous	FLT3 ITD hetero-zygous	FLT3 ITD-hetero-zygous, FLT3 D835H	FLT3 ITD-homo-zygous, WT1 S836*	FLT3 ITD-homo-zygous, WT1 S836*, D835H	FLT3 ITD-homo-zygous, FLT3 D835Y
VAF % by bulk sequencing	Pre-quizartinib	61.80	0.00	0.00	1.80	99.70						
	Relapse	84.60	2.50	11.00	7.40	99.70						
Population frequency % by SCS	Pre-quizartinib					7.20	21.54	46.82	0.0	1.10	0.00	0.00
	Relapse					7.24	54.88	7.53	9.9	6.38	6.43	2.91

*Bulk sequencing cannot determine zygosity
**Bulk sequencing cannot determine co-mutations.

Patient 7: bulk sequencing shows no co-mutations

Variant	Pre-Quizartinib Sample	Relapse Sample
	VAF% by bulk sequencing	
FLT3 D835V	0.00	31.00
FLT3 D835I	0.00	4.90
FLT3 D835F	0.00	3.80
FLT3 S838P	0.00	30.00
FLT3 ITD #1	11.80	19.30
FLT3 ITD #2	10.30	9.20
DNMT3A R882H	46.40	48.50

Patient 7: SCS

Time Points	Population Frequency (%)												
	WT	DNMT3A R882H	DNMT3A R882H, FLT3 D835V	DNMT3A R882H, FLT3 ITD #1	DNMT3A R882H, FLT3 ITD #2	DNMT3A R882H, both ITD	DNMT3A R882H, FLT3 both ITD, FLT3 D835V, FLT3 S838P	DNMT3A, FLT3 D835F	DNMT3A, FLT3 D835I	DNMT3A, FLT3 D835V, FLT3 S838P	DNMT3A, FLT3 ITD #1, FLT3 D835V, FLT3 S838P	DNMT3A, FLT3 ITD #2, FLT3 D835V, FLT3 S838P	DNMT3A, FLT3 ITD #1, FLT3 D835V
Pre-quizartinib	9.48	57.74	0.00	10.48	6.56	15.71	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Relapse	3.32	4.22	3.83	2.22	0.45	1.16	15.29	3.90	4.15	28.91	24.79	4.19	3.57

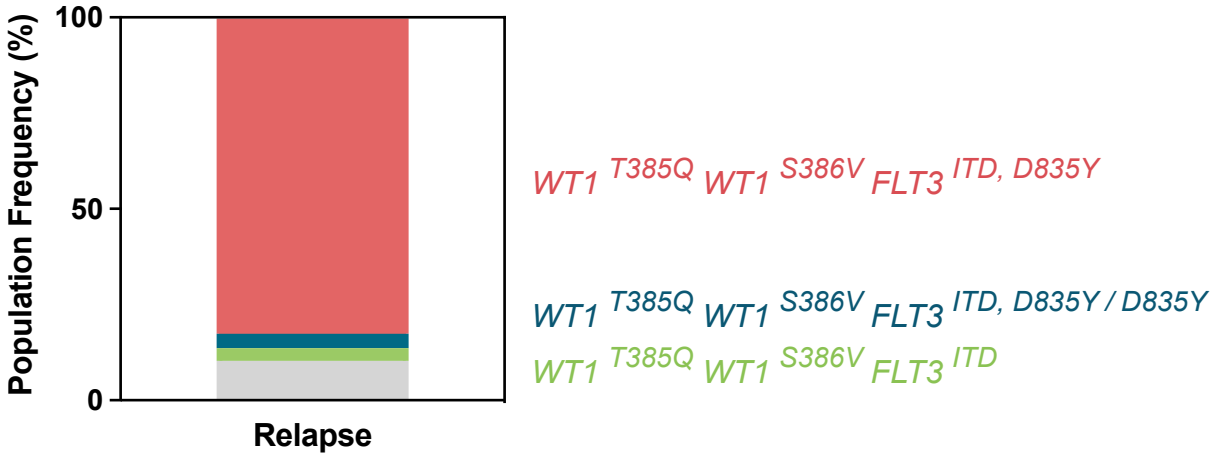
Patient 8

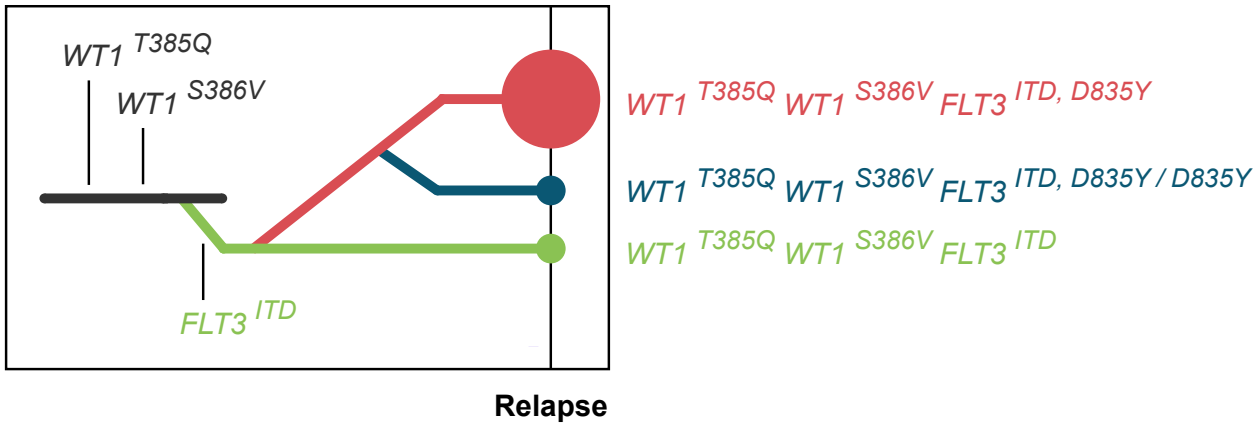
	Time Points	WT	FLT3 ITD	FLT3 D835Y	WT1 385Q	WT1 386V	FLT3 ITD, D835Y, WT1 385Q, WT1 386V	FLT3 ITD, D835Y homo-zygous, WT1 385Q, WT1 386V	FLT3 ITD, WT1 385Q WT1 386V
VAF % by bulk sequencing	Relapse		48.8	41.2	44.9	43.7			
Population frequency % by SCS	Relapse	10.31					82.37	3.75	3.29

Supplementary Table 3. FLT3 Internal Tandem Duplication (ITD) mutations.

Patient ID	ITD (if >1)	ITD location	ITD sequence
1	#1	chr13:28608278	TTTCTCTTGGAAACTCCCATTTGAGATCATATTCATATTC
	#2	chr13:28608300	CTTAGATGATTCTCTGAA
3	#1	chr13:28608262	CCAAACTCTAAATTTTCTCTTGGAAACTCCCATTTGAGATCATATTCATATTCTCT
	#2	chr13:28608305	CAGTTTCTCTTGG
4		chr13:28608297	CGCCTCAAACTCTAAATTTTC
5		chr13:28608624	TCGGGACTCTAAATTTTCTCTTGGAAACTCCCATTTGAGATCATATTCATATTC
6		chr13:28608308	TACCAAACTC
7	#1	chr13:28608267	AGCACCTGATCCTAGTACCTTCCCTGCAAAGACAAATGGTGAGTACGTGCA
	#2	chr13:28608104	TGCAGAAACATTTGGCACATTCCATTCTTACCAAACTCTAAATTTTCTCTTGGAAACTCCCATTTGAGATCATATTCAT
8		chr13:28608305	GATATTCTCTGAA

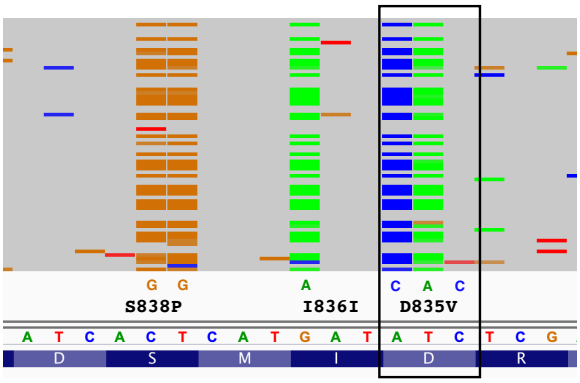
Supplementary Figure 1. Single cell sequencing of relapse sample from patient 8. Patient 8, for which only the relapse sample was able to be sequenced, demonstrates two different off-target mutations in the WT1 gene (in adjacent proteins) and heterozygous as well as homozygous D835Y mutations. The patient relapsed with a predominance of D835Y mutation in a FLT3-ITD^{+} allele.


Population Frequency (%)
100
50
0
Relapse
WT1 T385Q WT1 S386V FLT3 ITD, D835Y
WT1 T385Q WT1 S386V FLT3 ITD, D835Y / D835Y
WT1 T385Q WT1 S386V FLT3 ITD


WT1 T385Q
WT1 S386V
FLT3 ITD
Relapse
WT1 T385Q WT1 S386V FLT3 ITD, D835Y
WT1 T385Q WT1 S386V FLT3 ITD, D835Y / D835Y
WT1 T385Q WT1 S386V FLT3 ITD

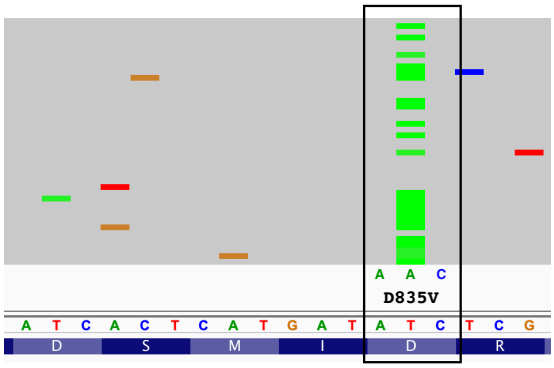
Supplementary Figure 2. Integrative Genomics Viewer (IGV) views from patient 7. **A**. Multi-nucleotide variant (MNV) changes to make D835V mutation. **B**. Single nucleotide variant (SNV) change to make D835V mutation. **C**. Population of D835V mutants from (B) with SNV gain a second mutation becoming MNV to make D835F. **D**. Population of D835V mutants from (B) with SNV gain a second mutation becoming an MNV to make D835I.

A.



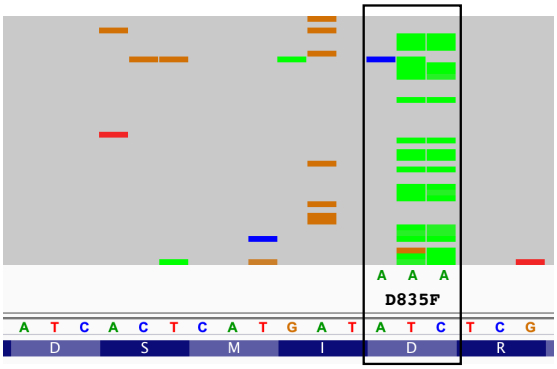
Codon: ATC > CAC
Reverse complement: GAT > GTG
Amino acid: D > V
D835V

B.



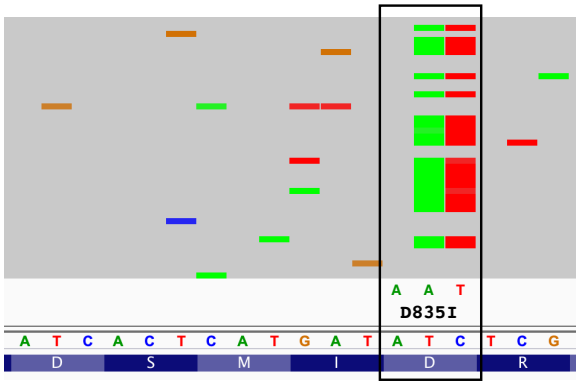
Codon: ATC > AAC
Reverse complement: GAT > GTT
Amino acid: D > V
D835V

C.



Codon: ATC > AAA
Reverse complement: GAT > TTT
Amino acid: D > F
D835F

D.



Codon: ATC > AAT
Reverse complement: GAT > ATT
Amino acid: D > I
D835I